The Effect Mechanism of Moist-Chilling and GA_3 on Seed Germination and Subsequent Seedling Growth of *Ferula ovina* Boiss

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Abstract: The germination of *Ferula ovina* seeds faces certain problems. The present research was designed to study the promotion of the germination of *Ferula ovina* seeds by moist-chilling and GA_3 applications. The results showed that *Ferula ovina* seeds display an endogenous dormancy that can be released by moist-chilling treatment for a certain period. In this respect, the best treatment was moist-chilling for 6 weeks at 5 ± 1 °C or for 4 weeks of moist-chilling followed by soaking in 500 ppm GA_3 solution for 24 h. These treatments significantly increased germination percentage and decreased time to 50% germination (T_{50}) compared to control. Also, the characteristics of the obtained seedlings were much better than those of control. Moreover, the 6-week moist-chilled seeds contained the highest soluble protein concentration. The combination between GA_3 and moist-chilling treatments produced different effects on seed germination, soluble protein depending on the length of the moist-chilling period. GA_3 application on un-chilled seeds improves the germination process. The concentration of soluble inorganic phosphorus of the tested seeds was negative (r = 0.88, $p<0.05$) while, the concentration of soluble organic phosphorus positively (r = 0.93, $P<0.05$) correlated with the germination percentage. It was concluded that treatment of moist-chilling for 6 weeks or 4 weeks followed by 500 ppm GA_3 is recommended for promoting the germination process of *Ferula ovina* seeds and improving growth characteristics of the subsequent seedlings.

Key Words: *Ferula ovina*, seed dormancy, protein analysis, phosphorus concentration, growth characteristics of seedlings.

INTRODUCTION

Genus of *Ferula* belongs to tribe Peucedaneae and subfamily of Apioideae and Umbelliferae family [1]. About 30 *Ferula* species are distributed in different regions of Iran [2].

Ecological studies show that the best habitats of the above-mentioned plants are the shaded north-facing slopes with an altitude of 2000-4000 m above sea level that have deep calcareous and well drained soil. The survey shows that ecological parameters (rainfall, fog and altitude) affect the quality of produced resins [1, 2]. *Ferula* plants can be consumed fresh or processed in several forms. Leaves and essential oils in *Ferula narthex, Ferula ovina* and *Ferula oopoda* traditionally have been considered to have medicinal value and there is strong evidence of antimicrobial active compounds [3]. Some of species have consumed for resins or as grazing plant.

Ferula ovina Boiss. is a forage species in this genus. Due to regular cutting and over exploitation, its natural spreads have become endangered in Iran. Seeds of this species suffer from dormancy and the possibility of propagation and the natural regeneration of this species through seed are very poor. The dormancy characteristic and optimum conditions for seed germination of this species have not been explained so far. Thus finding of some information about effective factors in dormancy breaking and optimal condition of seedling growth are necessary for recovery of natural spreads of this plant.

Seed dormancy is regarded as the failure of an intact viable seed to complete germination under favorable conditions. The release from dormancy can be triggered by a variety of environmental and chemical stimuli, is mediated through a common signal transduction chain that coordinates diverse cellular responses but that may differ between the seeds of different species and dormancy types. It has been suggested that there are related or common receptors for dormancy breaking agents within the plasma membrane of the responsive embryonic cells. When triggered, these receptors then initiate a signal transduction cascade, perhaps involving synthesis of or sensitization to germination-promoting gibberellins that lead to the completion of germination. Changes in the phosphorylating activity of membrane-associated, Ca^{+2}-dependent protein kinases that lead to dormancy or germination have been proposed as well [4]. In physiological dormant seeds, it is thought that temperature and gibberellins can both release dormancy and promote germination [5, 6].

Various dormancy breaking and germination stimulating treatments have been tried with seeds of many species of Apiaceae such as *Osmorhiza* [7, 8] ،*Ptilianium nuttalli* [9] ،*Perideridia gairdneri* [10], *Apium graveolens* [11] and *Ferula ovina* [12-16]. In this respect, gibberellic acid and moist-chilling treatments seem the most promising in many species of Apiaceae (ISTA) such as *Ferula ovina* [14]. The aim of the present study is to find a practical method to promote *Ferula ovina* seed germination and the subsequent

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seedling growth by means of moist-chilling and GA_3 applications and investigation of mechanism of effects of these treatments. Therefore, we examine the effect of such treatments on the biochemical changes associated with the germination of the treated seeds.

MATERIALS AND METHODS

Ferula ovina seeds were collected by research institute of forests and rangelands center of Isfahan, Iran in the year 2003. Seeds were immediately washed with tap water, divided into 8 groups (60 seeds for each). Each group was divided into 4 replicates (15 seeds for each) and subjected to one of the following treatments: soaking into tap water only for 24 h (control, Tc); soaking in a gibberellic acid (GA_3) solution at 500 ppm for 24 h (T1); 2-week moist-chilling at 5 ± 1 °C (T2); 2 week moist-chilling at 5 ± 1 °C followed by soaking in 500 ppm GA_3 solution for 24 h (T3); 4-week moist-chilling at 5 ± 1 °C (T4); 4-week moist-chilling at 5 ± 1 °C followed by soaking into 500 ppm GA_3 solution for 24 h (T5); 6-week moist-chilling at 5 ± 1 °C (T6); and 6-week moist-chilling at 5 ± 1 °C followed by soaking in 500 ppm GA_3 solution for 24 h (T7).

I) Seed Germination

After 4 weeks from sowing, the germination percentage was calculated weekly until the eighth week. Time (in days) to obtain 50% germination referred to as T_{50} [17] was also calculated using the following formula:

$$T_{50}=[(t_2-t_1)\times 50\%+(p_2t_1-p_1t_2)]/(p_2-p_1)$$

where t_1 = time at which the germination percentage is less than 50%, t_2 = time at which the germination percentage is more than 50%, and p_1 and p_2 are the measurements of germination percentage occurring at t_1 and t_2, respectively.

II) Seedling Characteristics

Eight 1-month-old seedlings were randomly collected from each treatment (two seedlings/replicate) and used for the measurements of seedling length (cm), leaf area (cm^2), dry weight of both seedling and root (g) and total chlorophyll concentration.

III) Phosphorus Concentration

The extraction and measurements were done according to Humphries [18] and the modifications of Hasaneen [19]. For each treatment 10 seeds were dried and ground, and then a sample of 0.5 g was homogenized with a solution of trichloroacetic acid (8%, w/v) in the presence of 2.0 g pure coarse sand. The resulting macerate was centrifuged at 5000 rpm for 5 min. This extraction process was repeated four times on the same sample and the resulting supernatants were collected, filtered and used to determine soluble inorganic and organic phosphorus contents spectrophotometrically at 710 nm.

IV) Soluble Protein Concentration

About 1.5 g of embryo tissues from each treatment was homogenized in a mortar and pestle with extraction buffer containing 10 ml of 0.15M NaCl, 1% 2-β-mercaptoethanol and 60mM Tris-HCl (pH=8.3) as described by Dure and Chillan [20] and Mahhhou and Dennis [21]. The homogenate was shaken for 5h at room temperature (about 20ºC) and centrifuged at 10000 rpm for 15 min. The insoluble residues were re-hemogenized with 5 ml of the previous extraction buffer and centrifuged. The protein content was measured spectrophotometrically at 595 nm according to Braford [22] and calculated on a dry weight basis.

STATISTICAL ANALYSIS

The obtained data were statistically analyzed as a factorial experimental design by SAS software [23] applying the least significant difference (LSD) at 5% for the comparison among the treatment means. Duncan's new multiple range tests and regression analysis were also used.

RESULTS

I) Seed Germination

Soaking seeds in 500 ppm GA_3 solution without moist-chilling significantly stimulated germination of seeds compared to controls after 6 weeks from sowing (Table **1**). Also after 4, 5, 6 and 7 weeks after sowing, all tested moist-chilling treatments with the different periods (2, 4 and 6 weeks) significantly accelerated seed germination and resulted in higher germination percentage than non-chilled and GA_3 treatments (Table **1**).

Response of chilled seeds to GA_3 treatment was dependent on the length of the moist-chilling periods. Subjecting the 4-week moist-chilled seeds to GA_3 treatment resulted in a significantly higher germination percentage than solely moist-chilling for the same period. While, subjecting the 2-week moist-chilled seeds to GA_3 treatment has no significant effect on the germination percentage compared with the same period of moist-chilling alone (Table **1**). In 2 week moist chilled seeds, up to the seventh week from sowing there is no significant difference in germination percentage between GA_3 treated seeds and GA_3 un-treated seeds. However, at the eighth week, the GA_3 treated seeds gave significantly higher germination percentage than 2 week moist chilled seeds alone (Table **1** and Fig. **1**).

The highest germination percentage (85%) and the lowest T_{50} (28 days), after 8 weeks from sowing, resulted from the 6-week moist-chilled seeds or 4-week moist-chilled + GA_3 (Fig. **1**). The differences between these treatment and the other tested treatments were significant. Increasing the moist-chilling period to longer than 6 weeks has no significant effect on the increasing of germination percentage and T_{50} (Fig. **1**).

Results after 8 weeks from sowing, showed that subjecting seeds to moist-chilling treatment for 6 weeks either alone or in combination with GA_3 resulted in significantly higher germination percentages (80 and 85%, respectively) than all of the treatments except of moist-chilling for 4 weeks in combination with GA_3 (Table **1**).

II) Seedling Growth

Generally, GA_3 and moist-chilling treatments, either alone or in combination, significantly improve seedling characteristics including seedling length, leaf area, root dry weight, total dry weight and total leaf chlorophyll compared with those of the control (Table **2**). The combination between GA_3 and moist-chilling treatments produced seedlings of significantly higher vegetative growth vigor than those of

Table 1. The Effect of GA_3 (500 ppm) and Four Moist-Chilling Periods Either Alone or in Combination on Germination Percentage

Treatments		Seed Germination% (Weeks from Sowing)				
Moist-Chilling (Week)	GA_3 (ppm)	4 Week	5 Week	6 Week	7 Week	8 Week
0	0	0e	5f	11e	21f	25 e
0	500	0e	12ef	23d	32d	33d
2	0	7d	20cde	38c	47c	50c
2	500	9cd	27cd	45bc	51c	59b
4	0	11c	34c	55b	62b	63b
4	500	19b	59a	67a	77a	79a
6	0	18b	47b	71a	74a	80a
6	500	32a	68a	77a	81a	85a
F-Test	***	***	***	***	***	***
$LSD_{0.05}$ Moist-Chilling*GA_3		5.4	11.1	12	9.2	8.0

Values within each column followed by the same letter are not statistically different at 5% level. *** Significant at level $P = 0.001$.

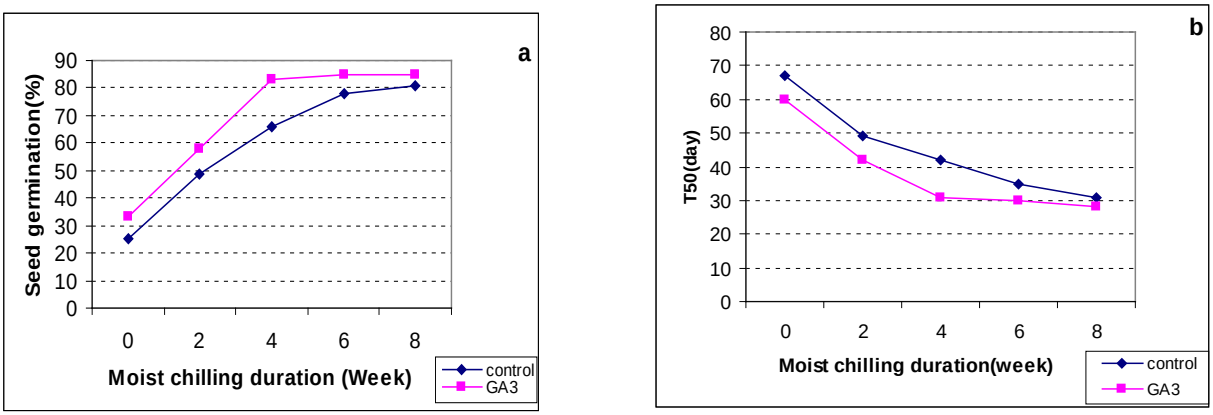

Fig. (1). Germination percentage (**a**) after 8 weeks from sowing and time (in days) to 50% germination (T_{50}) (**b**) of *Ferula ovina* seeds as affected four moist-chilling periods either alone(control) or in combination by GA_3 at 500 ppm.

GA_3 treatment alone. Increasing the moist-chilling period over 4 weeks had no significant effect on the seedling characteristics. Four-week moist-chilling treatment significantly surpassed the 2-week moist-chilling treatment in leaf area, root dry weight and total dry weight of the obtained seedling, seedling length and total chlorophyll but 6-week moist-chilling treatment has no more effect on growth parameters (Table **2**). Seedling length and total chlorophyll decrease after 6-week moist-chilling treatment but this effect was not significant. The invigorating effect of GA_3 treatment on the subsequent seedling growth was significantly lower than that of the tested moist-chilling periods.

III) Phosphorus Concentration

Generally, the tested treatments significantly decreased the soluble inorganic phosphorus concentration and significantly increased soluble organic phosphorus concentration of seed embryos compared with the control (Table **3**). Treating the 2-, 4-, or 6-week moist-chilled seeds with GA_3 led to a significant decrease in inorganic phosphorus and a significant increase in organic phosphorus compared to GA_3 treatment alone. Among all treatments, the 4-week moist-chilled seeds followed by GA_3 soaking expressed the lowest concentration of inorganic phosphorus and 6-week moist-chilled seeds followed by GA_3 soaking expressed the highest concentration of organic phosphorus. There were no significant differences in organic and inorganic phosphorus concentrations between the 4-week moist-chilled seeds and the 6-week moist-chilled seeds followed by GA_3. The 4-week moist-chilled and treated by GA_3 seeds or 6-week moist-chilled seeds contained significantly higher soluble organic phosphorus concentration than the 2-week moist-chilled seeds. The concentration of soluble inorganic phosphorus of the tested seeds was negatively ($r^2 = 0.78$, $P<0.05$) and the concentration of soluble organic phosphorus was positively ($r^2 = 0.87$, $P<0.05$) correlated with the percentage of germination (Fig. **2**).

Table 2. The Effect of GA_3 (500 ppm) and Four Moist-chilling Periods Either Alone or in Combination on Characteristics of Seedlings of *Ferula ovina*

Treatments		Seedling Length (cm)	Leaf Area / Seedling (cm^2)	Root D.W./ Seedling (gr)	Total D.W./ Seedling (gr)	Chlorophyll mg/100 cm^2
Moist-Chilling (Week)	GA_3 (ppm)					
0	0	10.2 c	82.8 e	0.12 e	1.04 d	1.49 d
0	500	10.6 c	101.0 d	0.28 d	2.15 c	1.78 d
2	0	12.5 b	121.2 c	0.27 d	2.58 bc	2.26 c
2	500	12.7 b	139.6 b	0.40 bc	3.31 ab	2.37 c
4	0	14.2 a	135.3 b	0.41 bc	3.32 ab	2.84 b
4	500	14.9 a	159.3 a	0.45 ab	3.51 a	3.34 a
6	0	13.9 a	154.2 a	0.38 c	3.21 ab	3.01 a
6	500	14.5 a	162.5 a	0.48 a	3.69 a	3.12 a
F-Test	***	***	***	***	***	***
$LSD_{0.05}$ Moist-Chilling*GA	1.42	19.9	0.61	0.73	0.42	1.42

Values within each column followed by the same letter are not statistically different at 5% level. Measurements were taken on 1-month-old seedlings. *** Significant at level $P = 0.001$.

Table 3. Changes in Phosphorus and Protein Compositions (mg/g dw) in *Ferula ovina* Seeds as Affected by GA_3 at 500 ppm and four Moist-Chilling Periods Either Alone or in Combination

Treatments		Soluble Inorganic Phosphorus	Soluble Organic Phosphorus	Soluble Protein
Moist-Chilling (Week)	GA_3(ppm)			
0	0	2.95 a	0.6 d	14.12 d
0	500	1.27 c	1.89 c	28.90 c
2	0	1.65 b	1.57 c	27.65 c
2	500	1.04 d	2.26 b	39.91b
4	0	0.98 d	2.35 b	38.17 b
4	500	0.88 d	3.05 a	47.8 a
6	0	0.93 d	2.85 a	46.11a
6	500	0.89 d	3.15 a	46.50 a
F-Test		***	***	***
$LSD_{0.05}$ Moist-Chilling*GA_3		0.21	041	2.10

Values within each column followed by the same letter are not statistically different at 5% level. *** Significant at level $P = 0.001$.

IV) Soluble Protein Concentration

The control seeds contained the lowest soluble protein concentration in their embryos while the 4-week moist-chilled + GA_3 treated seeds contained the highest (Table **3**). The 4-week moist-chilled + GA_3 treated seeds significantly surpassed the other treated seeds and the 6-week moist-chilling has no more effect. Applying GA_3 after moist-chilling gave different results of soluble protein concentration depending on the length of the moist-chilling period. The 2-week moist-chilled + GA_3 treated seeds contained a higher soluble protein concentration (39.91mg/g dw) than those of the 2-week moist-chilled seeds (27.65 mg/g dw) and the control (14.12 mg/g dw). Also, applying GA_3 after 4-week moist-chilling caused higher soluble protein concentration than those of the 4-week moist-chilled seeds alone. But 6 week moist-chilling with or without GA_3 had no significant difference. The concentration of the soluble protein in the seeds was positively (r^2=0.89, $p<0.05$) correlated with the germination percentage (Fig. **3**).

DISCUSSION AND CONCLUSION

The results of this study show that *Ferula ovina* seeds display an endogenous dormancy that can be released by moist-chilling treatment for a certain period. The 6-week

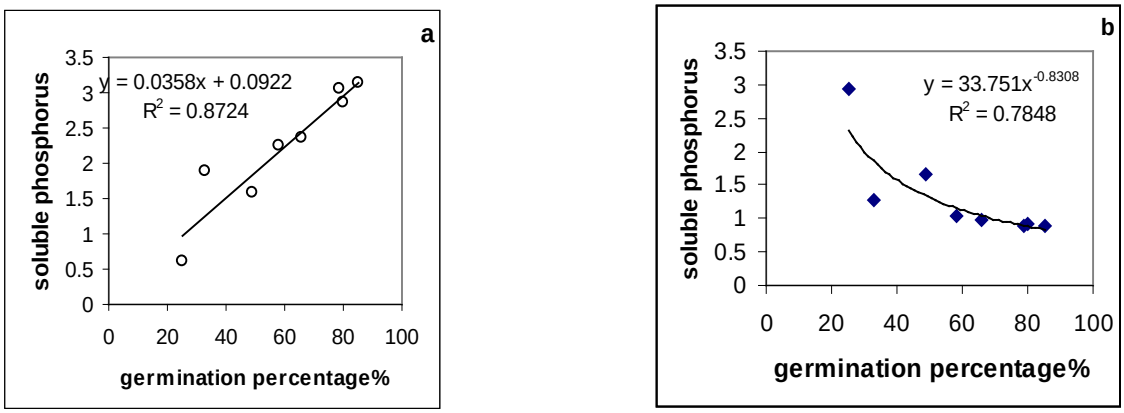

Fig. (2). Correlation between concentration of soluble organic (**a**) and inorganic (**b**) phosphorus and percentage of germination.

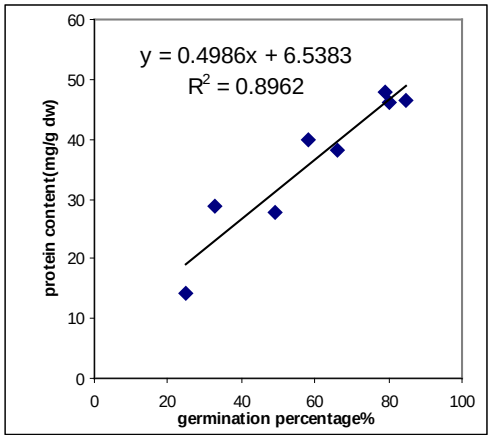

Fig. (3). Correlation between concentration of the soluble protein in the seeds and percentage of germination.

moist-chilled seeds compared with the other tested treatments had higher germination percentage and reducing T_{50}. The effect of cold treatment on seed dormancy breaking has also been confirmed in our previous research [12-15] and for other plants of Apiaceae such as: *Osmorhiza* [7, 8], *Ptilianium nuttalli* [9] *Anthriscus sylvestris* [24], *Perideridia gairdner i* [10], *Apium graveolens* [11]. Moreover, this treatment produced seedlings of better growth characteristics than control seeds (Table **2**). Such a result is in agreement with those reported by El-Nabawy *et al.* [25] on pecan seeds, by El-Dengawy [26] on peach seeds and by Samaan *et al.* [27] on apricot seeds. The 4-week moist-chilling treatment increased the level of soluble organic phosphorus and decreased the level of inorganic phosphorus compared with the control. In confirmation, El-Refaey and El-Dengawy [28] found that moist-chilling followed significantly increased soluble organic phosphorus of loquat seed embryo. These results show that chilling application affects phosphate metabolism in the seeds. Moist-chilling may increase the level of the organic phosphates like fructose 2,6-biphosphate [29] ATP [30] and nucleotides [26]. Such interpretation is in accordance with the finding of Khan *et al.* [31] who found a progressive increase in the synthesis of nucleic acids of pear embryos with the increase in the length of moist-chilling treatment. Also, during such treatment a significant increase in the levels of the pentose phosphate pathway enzymes coincides with dormancy breakage which may occur prior to seed germination [32, 33]. Moist-chilling application on peach seeds resulted in higher levels of uridine diphosphate (UDP) and thymine diphosphate (TDP) nucleotides and lower levels of both nucleosides and thymine monophosphate nucleotide (TMP) than non-moist-chilled seeds [26]. They postulated that such treatment encouraged the incorporation of these nucleosides and nucleotides in nucleic acids synthesis that needed for cell division of the embryo axis. The highest level of soluble inorganic phosphorus that coincided with the lowest level of soluble organic phosphorus in the control seeds might the attributed to a progressive release of inorganic phosphate within the cotyledons during the degradation of phosphate-containing compounds while its reutilization by the embryo axis is very slow [29].

Our results showed that moist-chilling treatments induced a great alteration in the level of seed soluble protein. This is strengthened by the findings of Hance and Bevington [34] on sugar maple seeds, Lin *et al.* [35] on pear seeds, El-Refaey and El-Dengawy [35] on loquat seeds and El-Dengawy [26] on peach seeds. Also, Mullen, *et al.* [36] reported that some changes in embryo mRNA populations and synthesized proteins occur during loblolly pine seed stratification, germination and postgerminative growth.

GA_3 application on un-chilled, in part, improve germination percentage and T_{50} during 6- 8 weeks after sowing (Table **1** and Fig. **1**). This result coincided with those of Baskin and Baskin [7] where they found positive effect for soaking in GA_3 solution on early germination *Osmorhiza claytonii* (Apiaceae) seeds. It was reported that GA_3 is effective in breaking the non-deep physiological dormancy, but it does not overcome the deep physiological dormancy [5, 24]. GA_3 effectiveness in stimulating of *Ferula ovina* seed germination might be referred to this possibility that *Ferula ovina* seeds have the non-deep physiological dormancy.

GAs appear not to be involved in control of dormancy per se but are rather important in the promotion and maintenance of germination, that is, they act after the ABA-mediated inhibition of germination has been overcome [4]. Two functions for GA during seed germination have been proposed. First GA increases the growth potential of embryo and promotes germination. Secondly, GA is necessary to overcome the mechanical restraint conferred by the seed covering layers by weakening of the tissues surrounding the radicle [37].

The combination between GA_3 and moist-chilling treatments produced differential effects on seed germination percentage, soluble proteins depending on the length of the moist-chilling period. GA_3 application after 2-week moist-chilling improved seed germination but it caused impressive effect on both germination percentage and T_{50} after 4- or 6-week moist-chilling. These results coincided with the alterations in protein level (Table **3**). Such results are in accordance with those of El-Dengawy [26] on peach seeds and of Chin *et al.* [38] on kiwi seeds. They concluded that the combination between a suitable moist-chilling period and an effective level of GA_3 would considerably enhance seed germination. GA treatment can break non-deep physiological dormancy and depending on species dormancy can also be broken by cold stratification or other treatments. Cold responses are mediated, at least in part, by promoting GA biosynthesis *via* enhanced expression of AtGA30x [6, 39]. Liu, *et al.* [40] showed that the blue micropylar EnD3 (BME3) GATA zing finger transcription factor is expressed in the radicle and seems to be involved as a positive regulator of seed germination and GA biosynthesis in response to cold stratification.

From the present results it can be concluded that treatment of moist-chilling for 6 or 4-week moist-chilling followed by soaking in 500 ppm GA_3 solution is recommended for promoting the germination process of *Ferula ovina* seeds and improving growth characteristics of the subsequent seedlings.

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